

Characterization of Covalently Bound Anti-Human Immunoglobulins on Self-Assembled Monolayer Modified Gold Electrodes

Brigitte Holzer, Kyriaki Manoli,* Nicoletta Ditaranto, Eleonora Macchia, Amber Tiwari, Cinzia Di Franco, Gaetano Scamarcio, Gerardo Palazzo, and Luisa Torsi

Bioconjugated gold surfaces constitute interesting platforms for bio-sensing applications. The immobilization of antibodies such as anti-immunoglobulin G and M (anti-IgG and anti-IgM) on gold electrodes via self-assembled monolayers (SAMs) is here studied as a model system for further immunoassays development. The biolayer is characterized by means of X-ray photoelectron spectroscopy (XPS), atomic force microscopy (AFM), a dedicated thin-film transistor (TFT)-based platform and electrochemical surface plasmon resonance (EC-SPR). XPS analysis confirms the presence of all the chemical species involved in the fabrication process as well as the covalent attachment of the antibodies with high reproducibility. Visualization of the biolayer topography by AFM shows nanostructures with a thickness consistent with the actual size of the protein, which is also verified by SPR measurements. EC-SPR allows taking advantage of complementary electrochemical and optical signals during the functionalization steps. Moreover, the functionalization of gold leads to a change in the work function, which is demonstrated in an electrolyte gated thin-film transistor configuration. Such configuration enables also to evaluate the electrostatic changes occurring on the gate that are connected with the threshold voltage shifts. The data support that functional biomodified gold surfaces can be reproducibly prepared, which is a prerequisite for further biosensor development.

1. Introduction

The attachment of biological recognition sites is essential for the successful development of reliable biosensors being able to detect target analytes at low concentrations. In addition, the immobilization strategy can affect biological systems and the processes associated with key relevant events such as biorecognition itself. The selected biofunctionalization protocol depends on several factors involving biomolecules properties, nature of the immobilization surface, sample matrix, and buffer medium. It also impacts on the sensor's analytical performance metrics such as the sensitivity, selectivity, and reproducibility. Ideally, the bioactive binding sites should be immobilized onto surfaces with controlled density and proper orientation in order to be accessible for reacting with the ligand. For a reproducible assay, bioreceptors activity and surface coverage should remain unchanged from one batch to the other. Up to date biomolecule immobilization involving silanized layers, polymer membranes, Langmuir–Blodgett films, protein

A, and self-assembled monolayer (SAM), has been achieved on various substrates, ensuring also the correct orientation of the recognition site without loss of activity and high loading of the sensor surface in order to gain maximum signal as well as selective analyte detection.^[1]

All the above reveal the importance of investigating the surface chemistry of biomodified materials, including noble metals, nanoparticles, metal oxides, polymers etc., for the optimization of the incorporation of biomolecules in miniaturized electronic sensors. Especially, the incorporation of biorecognition sites on inert metal surfaces such as gold gives rise to biosensors taking advantage of different physical quantifiable signals including electrochemical sensing,^[2–4] surface plasmon resonance (SPR),^[5–8] or the lately gaining significant attention electrolyte gated thin film transistors (EG-TFTs)^[9–13] based biosensors. Biorecognition elements (e.g., proteins, DNA, cells, etc.) have been largely immobilized on active gold sensor surfaces via weak physical (ionic, hydrophobic) or

Dr. B. Holzer, Dr. K. Manoli, Dr. N. Ditaranto, E. Macchia, A. Tiwari,
Prof. G. Palazzo, Prof. L. Torsi
Dipartimento di Chimica
Università degli Studi di Bari - "Aldo Moro"
Via Orabona 4, 70126 Bari, Italy
E-mail: kyriaki.manoli@uniba.it

Dr. N. Ditaranto, Prof. G. Palazzo, Prof. L. Torsi
CSGI (Center for Colloid and Surface Science) - Bari
Via Orabona 4, 70126 Bari, Italy

Dr. C. D. Franco, Prof. G. Scamarcio
CNR - Istituto di Fotonica e Nanotecnologie
Sede di Bari
Via Orabona 4, 70126 Bari, Italy

Prof. G. Scamarcio
Dipartimento di Fisica "M. Merlin" - Università degli
Studi di Bari - "Aldo Moro"
Via Orabona 4, 70126 Bari, Italy

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strong chemical (through thiol chemistry, bifunctional linkers or adapter molecules) interactions.^[14] Herein, a self-assembled monolayer approach was chosen to covalently link immunoglobulins to a gold electrode surface. The formation of covalent bonds between SAMs and the biological recognition sites was pursued taking advantage of the long-term stability of amide bonds exhibiting half-lives of approximately 600 years in neutral solution at 25 °C.^[15] Besides, thiol chemistry is among the most common routes to introduce a high number of functional groups on gold surface for covalent attachment of biomolecules with limited probability of denaturation.^[16]

Several surface analysis techniques are being employed to characterize quantitatively and qualitatively the attachment of proteins on a solid surface; X-ray photoelectron spectroscopy (XPS),^[17,18] atomic force microscopy (AFM),^[19] SPR,^[20] quartz crystal microbalance,^[21] grazing incidence X-ray diffraction,^[22] cyclic voltammetry (CV),^[23] and electrochemical impedance spectroscopy.^[24] The progress made in the development of biosensors recently also triggered the combination of these techniques to obtain integrative and reliable information on the topography as well as the chemical composition of surfaces bearing immobilized biorecognition sites.^[21] In this work, complementary surface analytical techniques, XPS, AFM, CV, and SPR were used to fully characterize gold surfaces at the successive functionalization steps. A systematic study of the biofunctionalized surface is presented. Moreover, in situ EC-SPR measurements were carried out to monitor the bioconjugation of protein to the thiol-modified surface. The combination of these techniques permits to gather more reliable and complementary data to characterize the bilayer film. Thickness and surface coverage were estimated using different techniques. The data support that the proposed protocol ensures a reproducible and effective immobilization of biomolecules on gold surfaces.

2. Results and Discussion

2.1. Fabrication Protocol of Gold Bioconjugation

The functionalization of precleaned gold electrodes **1** was realized by a multistep biofunctionalization process given in **Figure 1**. Chemisorption of a mixture of 3-mercaptopropionic acid (3-MPA) and 11-mercaptoundecanoic acid (11-MUA) in a ratio of 10:1 ensured the grafting of SAMs **2** on gold lamina. A mixture of SAMs with different chain lengths is known to be preferable for large biomolecules effective immobilization, since biomolecules are able to attach to the surface without undergoing conformational changes due to steric hindrance and therefore an enhanced surface coverage can be obtained.^[25] Terminal amine groups on the antibody enable covalent binding to the carboxylic functions of the SAM activated with *N*-ethyl-*N'*-(3-diethylaminopropyl)carbodiimide (EDC)/sulfo-*N*-hydroxysulfosuccinimide sodium salt) NHS **3**. Anti-immunoglobulin G and M (anti-IgG and anti-IgM) were then introduced as recognition sites **4**. These antibodies were chosen as model systems for the development of a future immunoassay platform. The nonreacted activated acidic functionalities were blocked applying ethanolamine (EA) **5**.

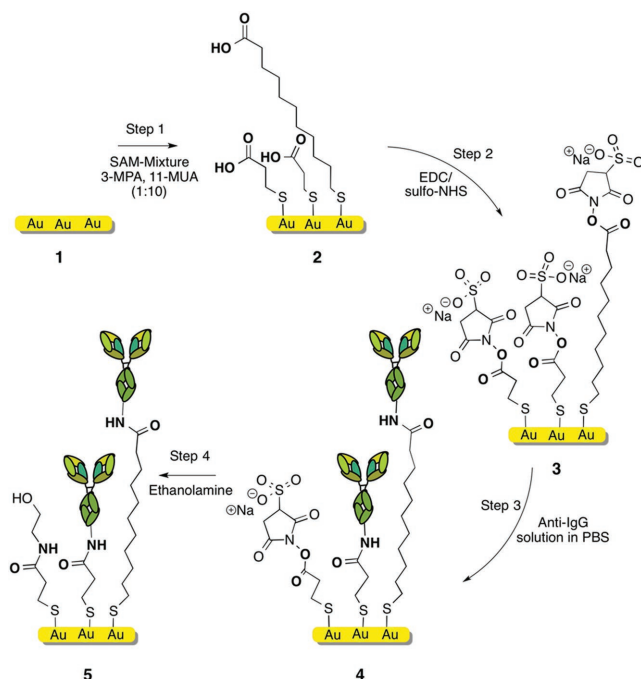


Figure 1. Stepwise functionalization toward biomodified gold electrodes.

2.2. XPS

XPS analysis was applied to characterize the surface composition of the modified gold electrodes in each step of the functionalization process. **Figure 2** shows the high-resolution XPS spectra measured for step 1–4 of the functionalization. The corresponding XPS survey spectra are given in Figure S1 (Supporting Information). The analysis of the shape of the background of the spectra, which is caused by inelastic scattered electrons at the near-surface structure, clearly shows the formation of a thin layer on the gold surface.^[26]

In **Table 1**, the atomic average percentages along with the standard deviation of the relevant elements obtained during the functionalization process based on three replicates are given. While a clear trend of increasing carbon and nitrogen content is apparent for after bioconjugation of the antibodies, a decrease is observable for the gold content, which is consistent with the attachment of a bilayer. The low standard deviation of the values of the atomic elemental percentages demonstrates the high reproducibility of this functionalization protocol.

The efficient coverage of a precleaned gold surface with carboxylic-acid-terminated alkanethiols after formation of the SAM monolayer is also confirmed by the S/Au and O/Au ratios (**Table 2**), which are distinctly above the ratios of the untreated sample confirming the presence of carboxylic acid terminated SAMs. It is found that the ratio for the untreated sample is 0.5 for C/Au and 1.8 for Au/C. These data correspond to a significantly lower carbon content compared to SAM-functionalized gold surfaces. Also, the formation of a covalent bioconjugated layer of biomolecules is evident, when considering that the Au/C, O/C, S/C, and N/C ratios remain comparable during the last two steps of the functionalization (attachment of anti-IgG and blocking with EA, **Figure 1**).

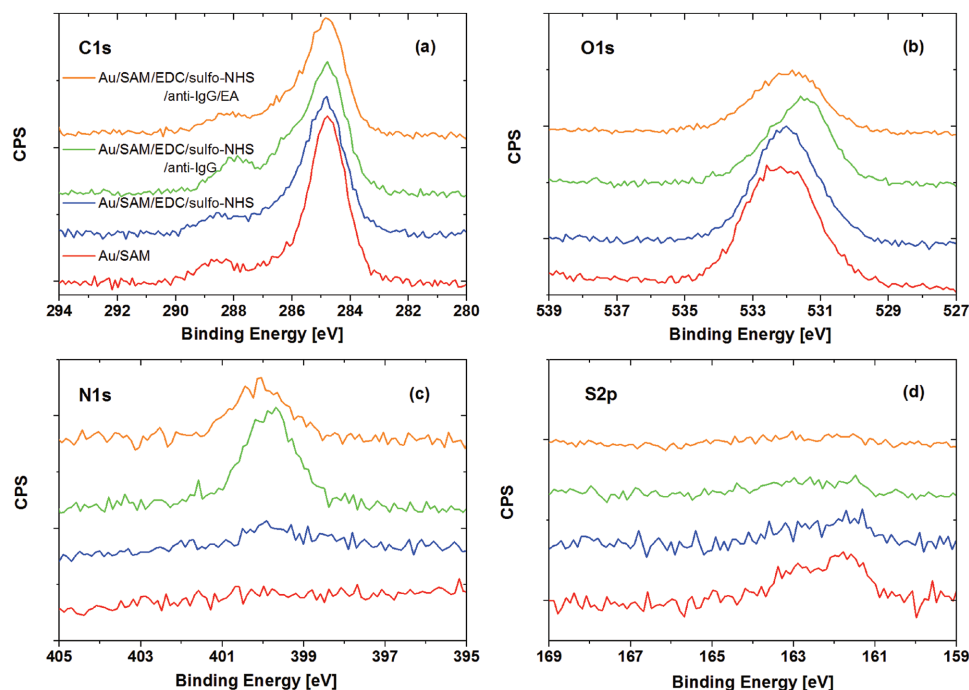


Figure 2. XPS high-resolution spectra of the a) C1s, b) O1s, c) 1Ns, and d) S2p core level of functionalized Au electrodes obtained for each functionalization step; SAM (red), activation with EDC/sulfo-NHS (red), immobilization of anti-human IgG (green) and blocking with ethanolamine (orange).

The analysis of the C1s core-level region confirms a main peak centered at 284.8 eV (Figure 3a), which can be assigned to the aliphatic carbons of the alkyl chains (CH_2 group). The attachment of the SAM-mixture molecules to the gold gate electrode is further evident by the peaks observed at 286.5 ± 0.1 and at 288.6 ± 0.1 eV, of which the former corresponds to emission from the carbon atom next to the sulfur atoms and the latter is characteristic for the carboxyl group of the SAM monolayer. After modification of the clean gold surface with SAM molecules, the high-resolution XPS spectra prove the presence of sulfur (Figure S2, Supporting Information). The S2p spectrum was deconvoluted by fitting the spin-orbit split doublet $\text{S}2\text{p}_{3/2}$ and $\text{S}2\text{p}_{1/2}$ in a ratio of 2:1. The position of this doublet at 162.0 ± 0.1 eV (Figure S2, Supporting Information) confirms the presence of a stable gold-sulfur bond (Au-S) formed by chemisorption of SAM thiols on the gold surface.^[27,28] Furthermore, since no detectable peaks above 163.5 eV were observed, it can be concluded that no unbound or oxidized thiol compounds are present on the gold surface, which is in agreement with the findings by Mendoza et al.^[29]

After the activation step of the carboxylic acid end groups applying EDC/sulfo-NHS, the presence of two sulfur species could be shown, which can be assigned on the one hand to the gold-sulfur bond of the SAM (162.0 ± 0.1 eV, Figure S2, Supporting Information) and on the other hand to the sulfonic acid salt of the applied sulfo-NHS (167.6 ± 0.3 eV, Figure S2, Supporting Information). During the activation step, XPS survey spectra also indicate the presence of nitrogen. The nitrogen binding energy is found in the high-resolution N1s spectrum at 401.8 ± 0.1 eV in Figure S2 (Supporting Information), which can be assigned to the succinimide ester intermediate formed by the reaction of EDC/sulfo-NHS with the carboxylic acid groups of the SAM and is in accordance to previous literature.^[30] A second observed nitrogen species at 399.6 ± 0.1 eV can be attributed to the presence of protonated amine of EDC.^[31]

A significant change in the C1s core-level spectrum can be observed after bioconjugation of the antibody anti-IgG to the activated SAM surface (Figure 3c). While the main peak at 284.8 eV can still be attributed to the aliphatic carbon atoms of the SAM and the immunoglobulin, the higher binding energy peaks can be ascribed to the peptide bonds of the immobilized

Table 1. Average chemical composition and corresponding standard deviation of Au, C, O, S, N of modified electrodes based on three replicates.

	Atomic composition (%)				
	Au	C	O	S	N
Au/SAM	34.0 ± 3.0	42.0 ± 4.0	22.6 ± 1.2	1.8 ± 0.5	–
Au/SAM/EDC/sulfo-NHS	21.4 ± 0.2	49.0 ± 3.0	23.4 ± 1.6	1.8 ± 0.6	4.3 ± 1.1
Au/SAM/EDC/sulfo-NHS/anti-IgG	15.0 ± 2.0	57.0 ± 2.4	18.3 ± 0.6	0.9 ± 0.5	9.4 ± 0.7
Au/SAM/EDC/sulfo-NHS/anti-IgG/EA	15.0 ± 3.1	58.2 ± 2.2	17.0 ± 1.1	1.0 ± 0.5	8.8 ± 1.0

Table 2. Intensity ratios calculated from average chemical compositions based on three replicates.

	C/Au	O/Au	S/Au	N/Au	Au/C	O/C	S/C	N/C
Au/SAM	1.24 ± 0.16	0.66 ± 0.07	0.05 ± 0.02	–	0.81 ± 0.11	0.54 ± 0.06	0.04 ± 0.01	–
Au/SAM/EDC/sulfo-NHS	2.29 ± 0.14	1.09 ± 0.08	0.08 ± 0.03	0.20 ± 0.05	0.44 ± 0.03	0.48 ± 0.04	0.04 ± 0.01	0.09 ± 0.02
Au/SAM/EDC/sulfo-NHS/anti-IgG	3.80 ± 0.53	1.22 ± 0.17	0.06 ± 0.03	0.63 ± 0.10	0.26 ± 0.04	0.32 ± 0.02	0.02 ± 0.01	0.16 ± 0.01
Au/SAM/EDC/sulfo-NHS/anti-IgG/EA	3.90 ± 0.82	1.13 ± 0.25	0.07 ± 0.04	0.59 ± 0.14	0.26 ± 0.05	0.29 ± 0.02	0.02 ± 0.01	0.15 ± 0.02

proteins: a peak at 286.1 ± 0.1 eV is attributed to C–N peptide bonds, while the last peak at 288.0 ± 0.1 eV is due to N–C=O bonds of peptide moieties of the protein (Figure S2, Supporting Information). Since the carboxylic carbon signal originating from the SAM is not detectable anymore and a clear shift of the higher energetic peak of carbon is evident (Figure 3c; Figure S2, Supporting Information), a highly efficient immobilization of proteins onto the activated SAM layer can be concluded. Also, the experimental data of the N1s core-level region recorded for the anti-IgG functionalized SAM prove the presence of only one nitrogen component at 399.8 ± 0.1 eV (Figure S2, Supporting Information), which can be assigned to the typical binding energy of the amide nitrogen atoms originating from the peptide functions of protein.

XPS data suggest the successful blocking of activated carboxyl groups with ethanolamine hydrochloride. The reactive sites are converted to corresponding ethylamides 5 (Figure 1), which is evident by the increase of the high energy peak O1s peak at 532.7 ± 0.1 eV (Figure S2, Supporting Information) originating from the terminal hydroxyl group of the attached ethanolamine.

Although an estimation of the surface coverage derived from the decrease of the XPS gold signal after the functionalization steps was not attempted, assuming that the coverage is

uniform the fact that the gold signal is still detectable after the functionalization process indicates that the average film thickness is lower than 10 nm, since the depth probed by XPS is at most 10 nm in normal emission.^[32]

Nevertheless, as previously mentioned the presence of a thin biolayer on the surface can be concluded from the shape of the background signal of the wide scan. The formation of a thin protein layer is consistent with the size of the antibody. X-ray diffraction measurements suggest a 3D structure of IgG antibodies with a size of about $14 \text{ nm} \times 8.5 \text{ nm} \times 3.8 \text{ nm}$,^[22] which can be also assumed for anti-IgG and anti-IgM.

2.3. AFM

The surface morphology of gold/SAM samples followed by bioconjugation using either anti-IgG or anti-IgM was evaluated by tapping mode AFM in air. Representative 3D surface topographies of gold-modified electrodes with SAM, SAM/anti-IgM and SAM/anti-IgG are shown in Figure S3 (Supporting Information). The dissimilar morphological surface features among the Au/SAM and Au/SAM/antibody images are indicative of successful surface modification. Chemisorption of SAM on the gold yields a flat surface, which is a prerequisite for the

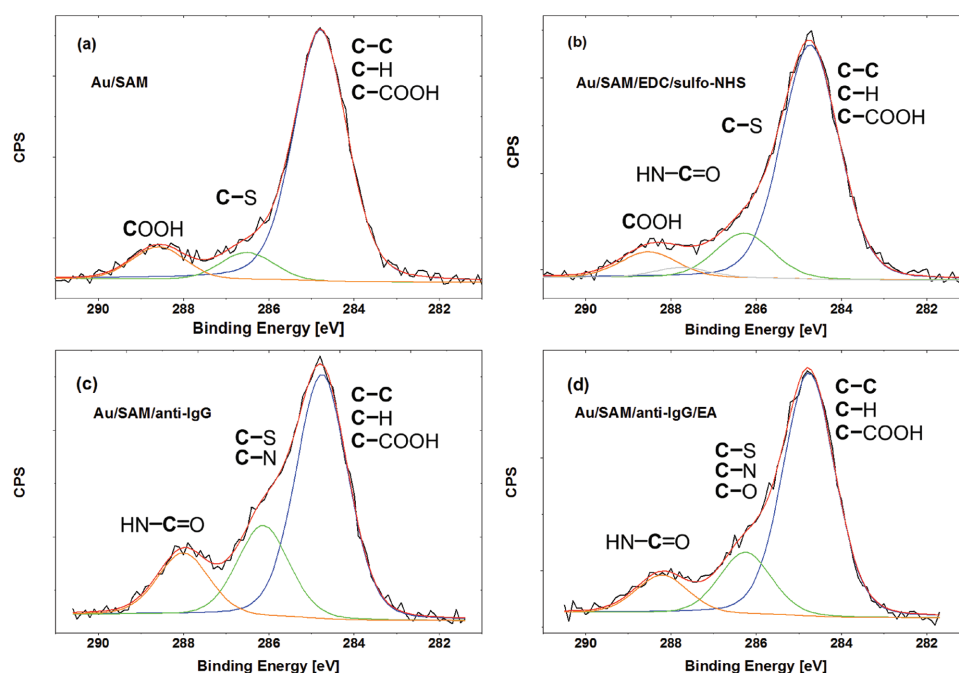


Figure 3. XPS spectra of the C1s core level of the modified Au electrodes: a) SAM formation on gold laminar, b) EDC/sulfo-NHS activation of carboxylic acid end groups of SAM, c) bioconjugation of anti-IgG, and d) blocking with ethanolamine.

Table 3. Surface roughness and average height of SAM-modified gold and after antibody immobilization are given as the average of five representative areas.

	Roughness [nm]	Height [nm]
Au/SAM	0.51 ± 0.07	14.9 ± 0.2
Au/SAM/anti-IgG	2.22 ± 0.28	22.3 ± 1.7
Au/SAM/anti-IgM	2.19 ± 0.41	23.0 ± 1.1

ordered immobilization of antibodies. Bioconjugation of antibodies leads to an increase in surface roughness, which is in good agreement with a layer of antibodies immobilized on the surface, however not necessarily with a preferential orientation. The average surface roughness of each sample is given in Table 3. Moreover, the average height as calculated from the 3D topographies of the three different surfaces allows estimating the thickness of the protein monolayer. The average height increased from ≈ 15 nm for Au/SAM, to ≈ 23 nm for the protein monolayer. The SAM thickness is expected to be few angstroms, however also the gold roughness contributes to this value. The difference of ≈ 8 nm between Au/SAM and the protein monolayer can be considered as a lower value for the anti-IgG and anti-IgM layer thickness. This value is compatible with the size of such large proteins^[22] and the results obtained by XPS analysis.

2.4. EG-TFT

TFTs have emerged as a new type of ultrasensitive, reliable, label-free (bio)sensors^[33–35] able to operate in aqueous media using the electrolyte-gated TFT configuration. They have been successfully exploited for detection of various clinical-related analytes such as proteins, DNA, hormones, etc. One of the most effective approaches to incorporate bioreceptors in an EG-TFT device involves biomolecules functionalization on the surface of the Au-gate electrode, through a self-assembled monolayer.^[9–11,36,37] Altering the gate surface in contact with the electrolyte by attaching bioreceptors can cause a shift in the threshold voltage of the transistor due to changes in the gate work function, particularly when charged species are involved. Herein, an EG-TFT device output current was measured at each step of the Au gate electrode functionalization process to assess the effect on the TFT characteristics. Figure 4 illustrates the I - V transfer curves obtained for bare Au, Au/SAM, and Au/SAM/anti-IgG/EA.

The obtained threshold values are -0.08 , -0.22 , and -0.28 V for Au, Au/SAM and Au/SAM/anti-IgG/EA, respectively. A shift of about 140 mV toward more negative values is observed for the TFT threshold voltage directly after the SAM chemisorption. This can be attributed to the dipole associated with the Au–S bond, which is known to be strong and oriented with its positive pole toward the gold electrode. Indeed, chemisorption of SAM leads to the modification of the work function of a gold metal surface, which can be due to the dipole formation at the metal–sulfur interface. Also, the dipole moments of the molecules within the SAM can contribute to the effect. Due to the electronegative terminal carboxylic group of the selected SAMs,

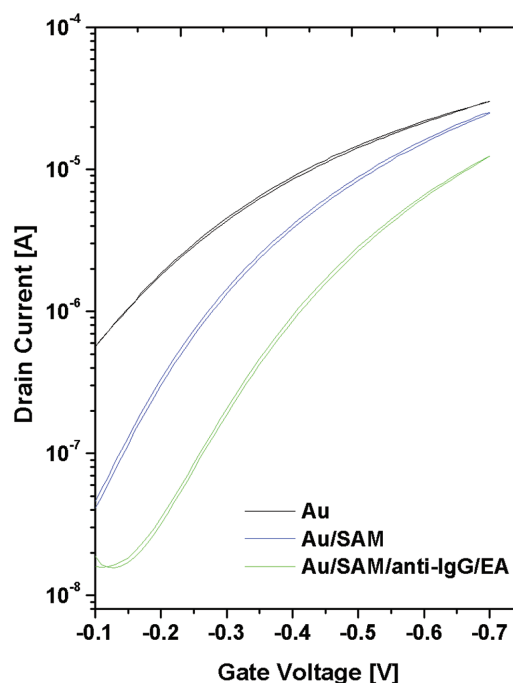


Figure 4. Transfer curve (I_{DS} vs. V_G) for EG-TFT with bare Au, Au/SAM, and Au/SAM/anti-IgG/EA as gate electrodes.

the two elicited dipoles have the same direction, toward the metal surface, presuming that the dipole vector points toward the positive pole.^[38]

Therefore, a net electric field opposite to the applied gate electric field is created, thus a more negative gate bias is required to turn on the TFT device compared to the bare Au electrode. Upon anti-IgG attachment, the threshold voltage becomes slightly more negative, shift of ≈ 60 mV. In a pH 6.9 measured for high-performance liquid chromatography (HPLC) water (i.e. pH between the isoelectric point of $(F_{ab})_2$ and F_c), the $(F_{ab})_2$ fragment is positively charged and the F_c negatively. Thus, the antibody can be modeled as an electric dipole where the dipole moment vector is pointing from the negatively charged F_c to the positively charged $(F_{ab})_2$ region.^[39,40] Although, controlled orientation of antibodies cannot be guaranteed, the further shift of the V_T may indicate an overall head on orientation for the majority of antibodies.

2.5. EC-SPR

The use of gold as a substrate in biosensing enables the possibility to take advantage of different physical quantifiable signals, which can be related also to the functionalization of the inert gold surface. On the one hand, the resonant oscillation of conduction electrons at the interface between a negative and positive permittivity material stimulated by incident light (used in surface plasmon resonance) can be employed to monitor surface-related phenomena. On the other hand, electrochemical experiments (e.g. cyclic voltammetry) allow monitoring the electron-transfer efficiency by detecting the change in faradaic response of a redox active species. Herein, both methods were

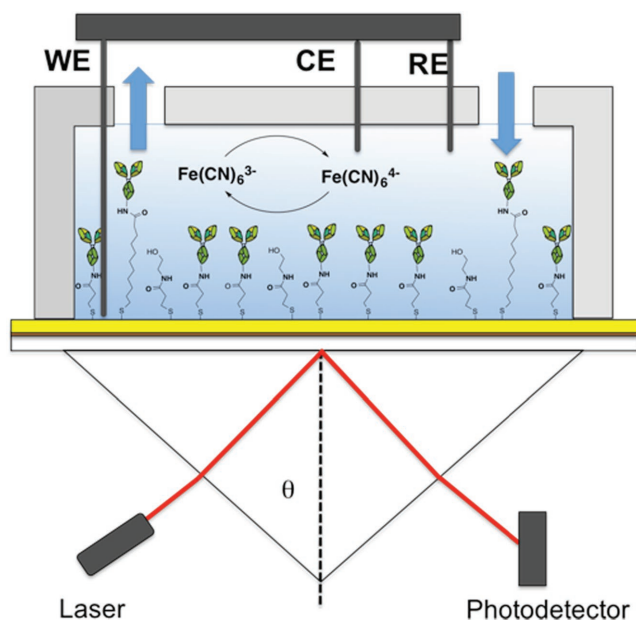


Figure 5. Schematic illustration of the EC-SPR measurement set-up.

used to characterize simultaneously the stepwise functionalization of gold toward a biointerface applicable as biosensor (Figure 5).

SPR measurements recording continuously the intensity of reflected light at a fixed angle were carried out during the immobilization process in a static flow cell. Representative SPR binding curves obtained for the immobilization of anti-IgM and anti-IgG are shown in Figure 6a and Figure S5 (Supporting Information), respectively.

A SAM-modified SPR gold slide was first activated with EDC/sulfo-NHS in water, which was observable as an increase in the SPR signal while a complete recovery of the signal is obtained after injecting water. In order to assure the native conformation of the immunoglobulin, the medium was changed from water to phosphate-buffered saline (PBS). After conjugation of both antibodies (anti-IgG as well as anti-IgM) to the activated surface a significant increase in the SPR signal could be observed.

Ethanolamine treatment was used to block any unreacted sites. While for the anti-IgG functionalized gold surface hardly any change in SPR signal could be observed, the signal for the anti-IgM gold surface decreases slightly. This may be explained by assuming the removal of physically adsorbed anti-IgM molecules after extensive rinsing.

It may be noted that in order to avoid nonspecific adsorption of antibodies on the gold electrode treatment of the functionalized surface with blocking reagents such as bovine albumin serum, polyethylene glycol derivatives, casein, tween 20, etc. may be employed to enhance the selectivity and sensitivity of the sensor.^[7,41]

In addition, the SPR angle scans were measured for Au/SAM before and after functionalization with anti-IgM at 670 nm. In order to determine the biolayer thickness,^[42] the SPR curves were fitted with Winspall 3.02 software using a multilayer model based on the Fresnel equation (Figure 6b).^[43] Initially, the thickness and apparent refractive index for pristine SPR slide (i.e. glass/Cr/Au) were determined. These values were used as reference for simulating the changes in thickness upon protein immobilization. The parameters obtained from the simulation of the SPR curves are presented in Table S1 (Supporting Information). The shift of the angle after the anti-IgM binding on the SAM/modified surface corresponds to an increase of ≈ 7 nm in thickness, which in good agreement with the protein layer thickness obtained from the topographical analysis.

The saturation response measured for protein adsorption was $8.3 \pm 0.7\%$ and $10.0 \pm 0.9\%$ reflected intensity change for anti-IgG and anti-IgM, respectively. Based on the reflected intensity change (ΔR) and by multiplying with 1500 ng cm^{-2} (conversion factor given by the manufacturer for protein in case of fixed angle measurements), the protein surface coverage was found to be $\Gamma_{\text{anti-IgG}} = 124 \pm 11$ and $\Gamma_{\text{anti-IgM}} = 151 \pm 29 \text{ ng cm}^{-2}$.

In situ CV and SPR measurements were carried out to monitor the immobilization of anti-IgM. The scale of electric isolation of the electrode surface was examined by using the ferri-/ferrocyanide redox probe, $[\text{Fe}(\text{CN})_6]^{3-/4-}$ over the potential range from -0.4 to 0.4 V with a scanning rate of 0.1 V s^{-1} . The functionalized SPR gold slide served as working electrode and a Pt electrode was used as pseudoreference electrode. Therefore, all potentials in this study are reported in comparison to a

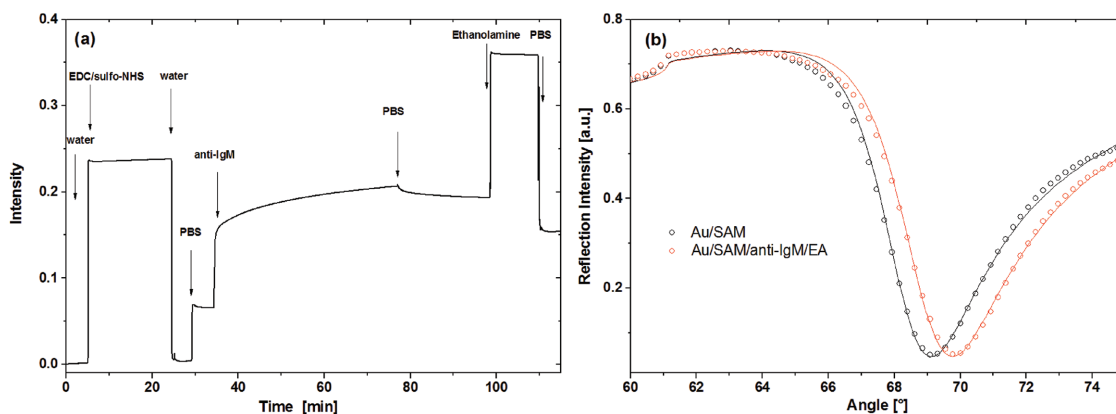


Figure 6. a) Sensogram for the bioconjugation of anti-IgM as well as after blocking with ethanolamine and b) experimental SPR angular reflectivity curves measured at a wavelength of 670 nm (circles) and corresponding fitted lines for a SAM (black) and antibody (red) modified SPR gold slide.

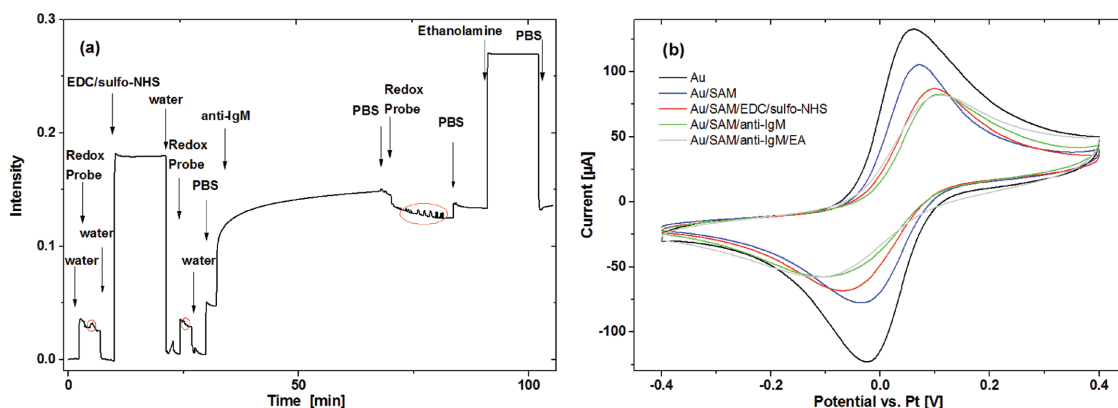


Figure 7. a) Sensogram for the bioconjugation of anti-IgM (red circles indicate events of in situ CV measurement) and b) in situ cyclic voltammograms for the functionalization of a SAM modified SPR gold slide with anti-human IgM; bare gold working electrode (black curve), SAM (blue), activation with EDC/sulfo-NHS (red), immobilization of anti-human IgM (green) and blocking with ethanolamine (gray).

Pt_{pseudo} potential. The sensogram along with the corresponding cyclic voltammograms of the gold surface modifications for each step is depicted in **Figure 7**, whereas the electrochemical data are given in **Table 4**.

A clear decrease in peak current of the ferrocyanide redox probe and an increase of the peak-to-peak distance from 86 to 108 mV could be observed after SAM chemisorption. The immobilization of anti-IgM leads to an even higher peak separation proving the efficiency of the applied immobilization protocol. Covalent attachment of the protein on the surface enhanced the insulating property of the electrode surface as can be seen by the further decrease of the faradaic currents.

In order to estimate the electroactive area of the electrode after bioconjugation, cyclic voltammetry was employed at different scan rates ν (20, 50, 100, and 200 mV s⁻¹) using the ferrocyanide redox probe (**Figure 8**). The relation between redox peak currents and square root of scan rate (**Figure 8c**) was employed to calculate the electrochemically active surface area by using Randles Sevcik equation

$$i_p = 0.4463nFAC \left(\frac{nF\nu D}{RT} \right)^{\frac{1}{2}} \quad (1)$$

with $T = 298$ K, $R = 8.314$ J K⁻¹ mol⁻¹, $F = 96480$ C mol⁻¹, $n = 1$, and D being the diffusion coefficient.

The obtained linear relationship between anodic peak current and the square root of the scan rate demonstrates a

Table 4. Electrochemical data (anodic peak potential E_{pa} , cathodic peak E_{pc} , and peak separation ΔE_p) measured for the bare Au, SAM-modified Au and anti-IgM immobilized.

	E_{pa} [mV]	E_{pc} [mV]	ΔE_p [mV]
Gold slide	60	-26	86
Au/SAM	70	-38	108
Au/SAM/EDC/sulfo-NHS	95	-68	163
Au/SAM/anti-IgM	105	-101	206
Au/SAM/anti-IgM/EA	108	-104	212

reversible reaction, in which the movement of the electroactive species to the surface of the electrode is diffusion controlled. Using the diffusion coefficient of $D = 6.5 \times 10^{-6}$ cm² s⁻¹ for Fe(CN)₆⁴⁻ in 0.1 M potassium chloride solution^[44] the unmodified surface area was calculated as 0.52 cm², which is consistent with the actual dimensions of the electrode surface (≈ 0.50 cm²) taking also surface roughness into account. After bioconjugation of anti-IgM to the electrode surface this value drops to 0.35 cm² indicating that the remaining electroactive surface is about 69%. Taking the difference of the active surface area before and after bioconjugation of anti-IgM as a measure for the area covered by the protein, we can quantify the amount of immobilized antibodies on the electrode. This results in a surface coverage of at least 31%. The mass of protein bound to the surface per unit area, was estimated equal to $\Gamma_{\text{anti-IgM/CV}} = 48 \pm 2$ ng cm⁻², assuming that the anti-IgM is resembled by a sphere with a radius of 7 nm.^[22,45] The value is lower than what was estimated with SPR, which can be attributed to the fact that the electroactive area does not necessarily match the geometrical area. This suggests that biolayer is permeable therefore allows the electron transfer to the electrode.

3. Conclusion

To conclude, a systematic investigation of biosensor like surfaces using XPS, AFM, EG-TFT, EC-SPR has been presented for all steps of biofunctionalization. A statistical chemical surface analysis of the modified gold electrodes in each step of the functionalization process indicated a successful bioconjugation of antibodies. Most importantly, the low standard deviation of the values of the atomic elemental percentages of different samples clearly demonstrated the high reproducibility of the functionalization protocol. Furthermore, the increase of background signal in XPS wide scan suggested the presence of a thin layer of immobilized antibodies, which was also confirmed by AFM and SPR measurements. In particular, the AFM analysis revealed distinct topographic surface features after the antibody bioconjugation of the SAM-modified electrode. The average height difference was ≈ 8 nm, while the r.m.s. roughness was ≈ 2 nm. The estimated thickness of the protein layer as obtained

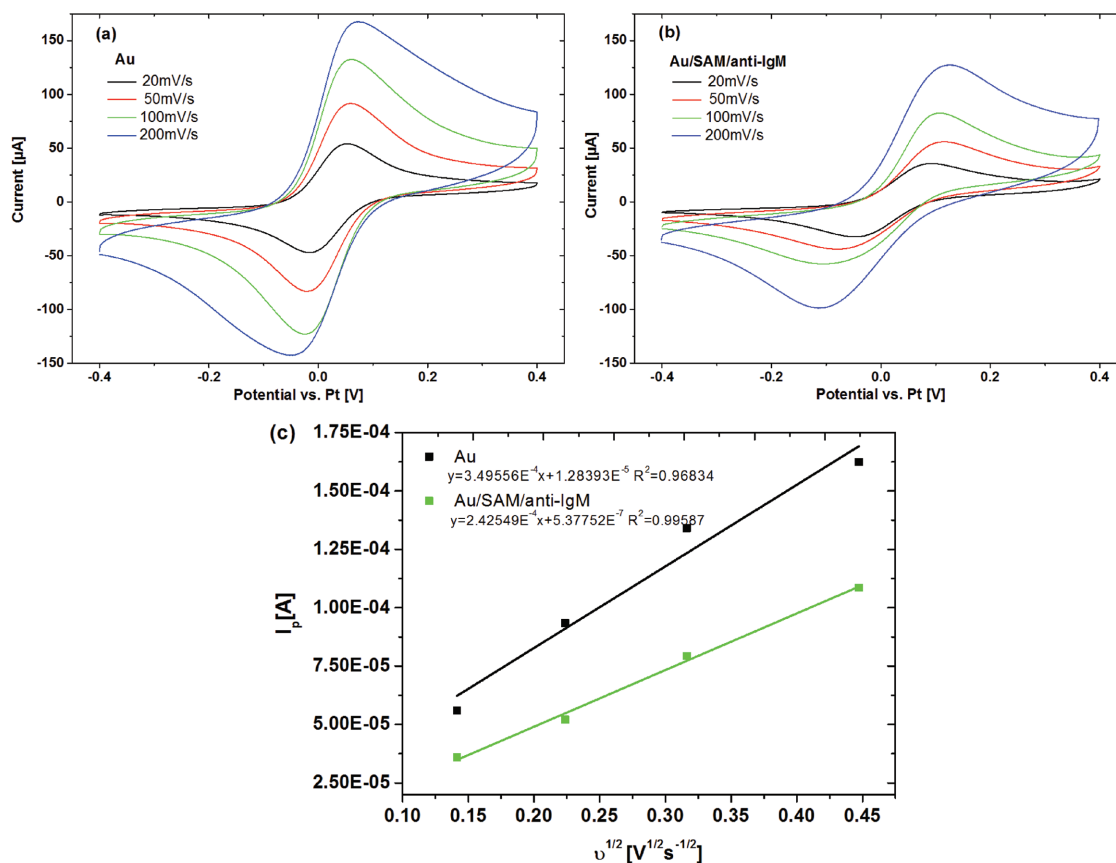


Figure 8. Cyclic voltammograms for a) bare gold and b) anti-IgM modified gold electrode obtained at different scan rates v (20, 50, 100, and 200 mV) using a solution of 1×10^{-3} M $K_4Fe(CN)_6$ in 0.1 M KCl electrolyte. c) Plot of anodic peak currents versus the square root of scan rate ($V s^{-1}$)^{1/2} for unmodified (black) and bioconjugated (green) gold electrodes.

from the SPR angle scan simulation was ≈ 7 nm. Employing the biomodified gold metal as gate electrode in EG-TFT configuration, a shift of the threshold voltage was observed and was correlated to preferable dipole orientation. In situ EC-SPR monitoring of the antibody immobilization demonstrated that both methods confirm a reproducible assay. Based on SPR measurements, the estimated surface coverage of anti-IgG and anti-IgM was ≈ 124 and ≈ 150 ng cm⁻², respectively, which corresponds to a coverage of 80% and 97% of the total surface. Finally, combined CV and SPR allow obtaining electrochemical and optical information when studying the adsorption of ultrathin protein layers on surfaces.

4. Experimental Section

Materials: 3-MPA ($\geq 99\%$), 11-MUA, EDC ($\geq 98\%$), sulfo-NHS ($\geq 98\%$), anti-human immunoglobulin G, anti-IgG, (Fc specific, 144 kDa), anti-human IgM (μ -chain specific), EA hydrochloride ($\geq 99\%$), and human IgG (150 kDa) were purchased from Sigma-Aldrich and used with no further purification. HPLC-grade water was purchased from Sigma-Aldrich. PBS buffer tablets were purchased from Sigma-Aldrich and PBS solution was prepared yielding a 0.01 M solution with pH 7.4.

Preparation of Functionalized Gold Electrodes: Gold laminar sheets (≈ 0.6 cm²) were cleaned using the following cleaning procedure: (1) a Bunsen burner was applied to remove organic residues, (2) the electrodes were immersed in a piranha solution (H_2SO_4/H_2O_2 , 3:1, v/v)

for 10 min, and (3) washed by immersion in boiling HPLC water for 10 min; then (4) UV/ozone surface cleaning was used as the last step.

The cleaned electrodes were used immediately afterward to chemisorb the self-assembled monolayer using a 10×10^{-3} M mixture of the thiols 3-MPA and 11-MUA in a 10:1 ratio prepared in absolute ethanol according to literature.^[25] The gold electrodes were immersed into the mixed SAM solution and kept in the dark under N_2 for 18 h at room temperature. After functionalization the SAM-modified gold sheets were rinsed with absolute ethanol and further activated by immersion in a water solution of 0.2 M EDC *N*-ethyl-*N'*-(3-diethylaminopropyl)-carbodiimide and 0.05 M sulfo-NHS (*N*-hydroxysulfosuccinimide sodium salt) for 2 h at room temperature. The activated SAM-functionalized gold sheets were rinsed with HPLC water and then either placed in a solution of anti-human IgG or anti-human IgM phosphate-buffer solution (100 μ g mL⁻¹) for 2 h at room temperature. Unreacted sites of the biofunctionalized gold laminar were converted to corresponding ethylamides using a 1 M ethanolamine hydrochloride solution in PBS for 1 h at room temperature.

X-ray Photoelectron Spectroscopy Analysis: X-ray photoelectron spectroscopy analyses were performed with a Versa Probe II Scanning XPS Microprobe spectrometer (Physical Electronics GmbH). The measurements were done in Large Area mode with a 100 μ m Al X-ray spot rasterized over a 1400 $\times$ 200 μ m area, at a power of 100 W. Wide scans and detailed spectra were acquired in fixed analyzer transmission mode with a pass energy of 117.40 and 46.95 eV, respectively. An electron gun was used for charge compensation (1.0 V 20.0 μ A). Data processing was performed using the MultiPak software v. 9.5.0.8.

Atomic Force Microscopy: The surface topography of modified electrodes was measured by means of an atomic force microscope

(AFM mod. NTEGRA Spectra, NT-MDT, Moscow, Russia) operated in a semicontact mode. The micrographs were recorded in air using free oscillation amplitude of 70 nm and scan step size of 2 nm. High-resolution single-crystal Si probes (mod. NGS01, NT-MDT) with a resonant frequency of 150 kHz, a force constant of 5 N m⁻¹, and a nominal tip radius of 6 nm, were used. The image analysis was performed by means of the Nova Px software (NT-MDT).

Electrolyte Gate Thin-Film Transistor: Interdigitated gold source (S) and drain (D) (50 nm, thick) were photolithographically defined on a Si/SiO₂ substrate, using titanium as adhesion promoting layer (5 nm). The distance between two differently biased fingers defines the channel length ($L = 5 \mu\text{m}$), while the channel width is $W = 1040 \mu\text{m}$. The S-D patterned substrates were cleaned in an ultrasonic bath of solvents of increasing polarity (acetone and isopropanol, respectively) for 10 min each and finally dried with nitrogen. Poly(3-hexylthiophene-2,5-diyl) (P3HT) (Sigma-Aldrich, regioregularity >99%) served as the semiconductor. The P3HT solution (2.6 mg mL⁻¹ in chlorobenzene) was spin-coated at 2000 r.p.m. for 20 s. The film was then annealed at 80 °C for 1 h. A polydimethylsiloxane well was glued around the interdigitated channels area and was filled with 300 μL of water (HPLC grade). The gate electrode was placed on top of the water gating medium. EG-TFTs were characterized with Agilent 4155C semiconductor parameter analyzer by measuring the transfer characteristics using bare, SAM and anti-IgG modified Au electrode as gate. The drain to source current (I_{DS}) was measured as a function of V_{G} (ranging from -0.1 to -0.7 V in steps of -0.01 V) at a constant drain voltage of -0.4 V.

Electrochemical Measurements and Surface Plasmon Resonance: A SPR Navi 200-L instrument equipped with two light sources (670 and 785 nm wavelength) and an electrochemical cell was used monitoring in situ surface functionalization. Gold-coated SPR slides (≈ 50 nm) with a chromium adhesion layer (≈ 2 nm) were obtained from BioNavis Ltd and cleaned using a piranha solution ($\text{H}_2\text{SO}_4/\text{H}_2\text{O}_2$, 3:1 v/v) for 10 min, washing by immersion in boiling HPLC water for 10 min and UV/ozone surface cleaning for 10 min. Gold slides were functionalized with SAM prior to SPR measurements according to the above given functionalization protocol. All further functionalization steps were realized by static injection at 22 °C of 300 μL solutions after docking the chip. For the electrochemical measurements, the gold SPR slide with an approximate area of 0.50 cm² was used as a working electrode (WE), while Pt was used as a pseudo-reference ($\text{RE}_{\text{pseudo}}$) and counter electrode (CE). All electrodes were directly connected to a CH Instrument Electrochemical Analyzer (Model CHI1230B). The anodic current is set positive. Cyclic voltammetry was carried out in the presence of 1×10^{-3} M potassium ferrocyanide $\text{K}_4[\text{Fe}(\text{CN})_6] \cdot 3\text{H}_2\text{O}$ (Sigma Aldrich, 98.5%) in 0.1 M potassium chloride (Fluka, puriss p.a.) with an applied potential in the range of -0.4 to $+0.4$ V.

Multilayer simulations for sample layer analysis (refractive index and thin-film thickness) were carried out by using the Winspall 3.02 software based on the Fresnel's equation.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

biosensors, immunoglobulin, self-assembled monolayers, surface analysis, thin-film transistors

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